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SHORT COMMUNICATION

PREGNENOLONE AND PROGESTERONE FROM 20α-HYDROXYCHOLESTEROL BY *CHEIRANTHUS CHEIRI* LEAF HOMOGENATES

S. J. STOHS and M. M. EL-OLEMY

College of Pharmacy, University of Nebraska, Lincoln, Nebraska 68508, U.S.A.

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Abstract—The incubation of 20α-hydroxycholesterol-7-^{3}H with leaf homogenates of *Cheiranthus cheiri* results in the formation of pregnenolone and progesterone. Preliminary results suggest that leaf homogenates of *Nerium oleander*, but not *Digitalis purpurea* or *Strophanthus kombe*, will yield the same two products under identical conditions.

INTRODUCTION

PRESENT knowledge of the biosynthesis of cardenolides suggests that the order of the metabolic reactions appears to be: cholesterol → pregnenolone → progesterone → cardenolides.[1-3] The conversion of cholesterol to prenenolone by leaves of *Digitalis purpurea*[1,4] and *Holarrhena floribunda*[5] has been observed. Wickramasinghe *et al.*[6] have simultaneously applied 20α-hydroxycholesterol-7-^{3}H and cholesterol-4-^{14}C to *D. lanata* leaves which were harvested after 30 days. Progesterone-7-^{3}H devoid of ^{14}C was isolated. The biosynthesized cardenolides that were isolated had markedly differing ratios of ^{3}H/^{14}C, suggesting that several routes might be operative in the biosynthesis of cardenolides from cholesterol.[6] The potential involvement of 20α-hydroxycholesterol in the conversion of cholesterol to pregnenolone in mammalian systems has been extensively investigated.[7] The incorporation of both pregnenolone and progesterone into cardenolides by various plants has been repeatedly demonstrated.[8-12]

We have examined the ability of leaf homogenates from cardenolide producing plants to cleave the side-chain of 20α-hydroxycholesterol-7-^{3}H, producing pregnenolone-7-^{3}H and progesterone-7-^{3}H.

RESULTS

Leaf homogenates of *Cheiranthus cheiri*, *Nerium oleander*, *Digitalis purpurea* and *Strophanthus kombe* were incubated with 20α-hydroxycholesterol-7-^{3}H in a buffer medium containing an NADPH generating system. The reaction mixtures were extracted, dried,

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concentrated, and the extracts were subjected to TLC. The radioactive zones corresponding to pregnenolone and progesterone were counted by liquid scintillation counting. The abilities of these leaf homogenates to produce side-chain cleavage of the 20α-hydroxycholesterol to yield pregnenolone, and subsequently progesterone are given in Table 1. The results are expressed as the percent recovered ^3H in the pregnenolone and progesterone fractions.

TABLE 1. PREGNENOLONE AND PROGESTERONE FROM 20α-HYDROXYCHOLESTEROL-7-^3H BY LEAF HOMOGENATES

Leaf source	Incubation time (hr)	% Radioactivity recovered as Pregnenolone	Progesterone
Cheiranthus cheiri	2	6·68 ± 1·95	2·56 ± 0·27
C. cheiri	4	8·68 ± 3·84	3·23 ± 0 49
Nerium oleander	4	1·48 ± 0·53	0·57 ± 0·11
Digitalis purpurea	4	0·0	0·0
Strophanthus kombe	4	0·0	0·0

The homogenate from 1 g of leaves was incubated at 30° for 2 or 4 hr on a shaker with 1·0 μc 20α-hydroxycholesterol in the presence of an NADPH generating system. The extraction and assay procedures are described under Experimental. Each value represents the average of 3–4 determinations with the standard deviation.

In 4 hr, 8·68% of the extracted radioactivity was present as pregnenolone and 3·23% present as progesterone following incubation with leaf homogenates of *C. cheiri*. As can be seen in Table 1, under identical conditions *N. oleander* produced significantly smaller amounts of both metabolites, while neither metabolite could be found, following incubation with leaf homogenates of *D. purpurea* and *S. kombe*.

The two metabolites from *C. cheiri* incubations were isolated by preparative TLC and co-crystallized to constant specific activity with the corresponding non-radioactive pregnenolone and progesterone. The results are given in Tables 2 and 3. In addition, pregnenolone was acetylated and subsequently recrystallized to constant specific activity as well. From the specific activities of the radiochemically homogenous pregnenolone and progesterone,

TABLE 2. CO-CRYSTALLIZATION OF PREGNENOLONE-7-^3H AND PREGNENOLONE ACETATE TO CONSTANT SPECIFIC ACTIVITY

Compound	Solvent	Counts/min/mg
Pregnenolone	50% EtOH	192 ± 5
Pregnenolone	$CHCl_3$–petroleum	151 ± 6
Pregnenolone	$CHCl_3$–petroleum	149 ± 9
Pregnenolone	$CHCl_3$–petroleum	158 ± 9
Pregnenolone acetate	MeOH	138 ± 11
Pregnenolone acetate	80% EtOH	140 ± 10

Pregnenolone-^3H, produced by side-chain cleavage of 20α-hydroxycholesterol employing *C. cheiri* leaf homogenates, was isolated by preparative TLC. The isolated pregnenolone-^3H was co-crystallized to constant specific activity. The acetate was also formed and re-crystallized to constant specific activity. Each value is the average of four aliquots with the standard deviation

TABLE 3. CO-CRYSTALLIZATION OF PROGESTERONE-^{3}H TO CONSTANT SPECIFIC ACTIVITY

Solvent	Specific activity (counts/min/mg)
95% EtOH	145 ± 16
50% EtOH	112 ± 8
CH_2Cl_2–MeOH–H_2O	113 ± 6
$CHCl_3$–petroleum	110 ± 5

Progesterone formed following the side-chain cleavage of 20α-hydroxycholesterol upon incubation with *C. cheiri* was isolated by preparative TLC, and co-crystallized to constant specific activity. Each value is the average of four aliquots with the standard deviation.

the per cent ^{3}H present in each was back-calculated with suitable corrections for aliquots removed for initial analyses. By these calculations, 9·5% of the recovered ^{3}H was present as pregnenolone and 3·4% was present as progesterone. These results agree well with those from the TLC method given in Table 1.

DISCUSSION

The results indicate that 20α-hydroxycholesterol can be converted to pregnenolone which is further metabolized to progesterone. If there are several routes operating simultaneously in the biosynthesis of cardenolides from cholesterol as has been suggested,[6] 20α-hydroxycholesterol may be an intermediate in one of these routes or it may be easily shunted into one or more of the biosynthetic pathways.

Not all plants studied were capable of metabolizing 20α-hydroxycholesterol under our experimental conditions. We have similarly observed that *C. cheiri* leaf and tissue culture homogenates are capable of metabolizing cholesterol[13] and sitosterol[14] while leaf homogenates of several other cardenolide producing plants fail to do so under identical conditions. Several explanations are possible. These plants may simply not produce high levels of these enzymes, and/or this reaction involving 20α-hydroxycholesterol may not be significant in these plants. In the homogenates from *Digitalis* and *Strophanthus*, a high level of plant phenols may inhibit the enzyme.[15] Therefore, the additives employed may not be suitable for all plants.

EXPERIMENTAL

Homogenates. 30% leaf homogenates of 4–6 month-old *Cheiranthus cheiri*, *Digitalis purpurea* and *Strophanthus kombe* were prepared in a buffer medium containing 0·05 M tris-chloride, 0·25 M sucrose, 0·005 M $MgCl_2$, 0·003 M L-cysteine HCl, 0·045 M mercaptoethanol, and 1 mg/ml bovine serum albumin fraction V (Sigma Chemical Co.). Leaves of greenhouse grown 10-yr-old *Nerium oleander* were similarly homogenized. A Sorvall Omni-Mixer Homogenizer, operating at maximum speed for two 20-sec intervals, was used to prepare the homogenates. All homogenizations were performed in an ice-bath. The homogenates were filtered through one layer of muslin in the cold, and the filtrates were used as the enzyme source.

Incubations. The 20α-hydroxycholesterol-7-^{3}H was obtained from New England Nuclear and had a specific activity of 9·3 c/mM. It was purified by TLC on silica gel H (Brinkmann) plates developed twice with

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isoPr_2O–petroleum–HOAc (90:30:1). The homogenate from 1 g of leaves was incubated with 1·0 μc 20α-hydroxycholesterol-7-^{3}H in a total volume of 5·0 ml homogenization buffer to which was also added 2·5 units glucose-6-phosphate dehydrogenase, 7·0 mg glucose-6-phosphate, and 1·5 mg $NADP^+$. The substrate was added in 0·10 ml 70% EtOH All incubations were conducted at 30° on a water bath shaker, aerating with 95% O_2–5% CO_2. Each reaction mixture was extracted with EtOAc–HOAc (100:1) after the desired incubation period.[14] The organic phase was dried over anydrous sodium sulfate and evaporated to dryness *in vacuo*. The extraction efficiency varied from approx. 50–90%.

Metabolite identification. Aliquots of the extracts containing 15,000–20,000 counts/min were co-chromatographed with the reference standards 20α-hydroxycholesterol, cholesterol, pregnenolone and progesterone on silica gel H plates which were divided into 2 cm wide columns. The plates were developed three successive times with *iso*-Pr_2O–petroleum–HOAc (70:30:1), allowing the plates to air-dry 15 min between developments. Iodine vapors were used to locate the reference standards. The radioactivity associated with each reference standard as well as the remainder of each column was counted employing the methods previously described.[14]

The two metabolites from 20α-hydroxycholesterol with R_fs corresponding to pregnenolone and progesterone were isolated by preparative TLC using 0·50 mm thick silica gel H plates and the solvents described above Extracts from 4 hr incubations were pooled and subjected to TLC. The areas corresponding to pregnenolone and progesterone were Soxhlet extracted for 24 hr with $CHCl_3$. Each metabolite was repurified by TLC in CH_2Cl_2–MeOH (97:3). The metabolite corresponding to pregnenolone was recrystallized with 200 mg non-radioactive pregnenolone from the solvents shown in Table 2. Approximately 20 mg of the pregnenolone-^{3}H, which had been recrystallized ($\times$4), was acetylated by dissolving in a mixture of 5 ml benzene, 5 ml Ac_2O and 0·5 ml pyridine, followed by refluxing for 1 hr. MeOH (10 ml) was added with subsequent evaporation under vacuum to near dryness. Distilled H_2O (10 ml) and 0·20 ml conc. H_2SO_4 was added. This mixture was subsequently extracted ($\times$3) with 10 ml $CHCl_3$. The $CHCl_3$ extracts were pooled, dried, and evaporated to dryness. The pregnenolone acetate was recrystallized from MeOH and 80% EtOH, and the results are given in Table 2.

The metabolite corresponding to progesterone was recrystallized with 100 mg non-radioactive progesterone. The solvents used and the results are given in Table 3.

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